FINAL REPORT

Study Title

DIRECT PEPTIDE REACTIVITY ASSAY (DPRA)

Test Article

JA900-DAA

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Study Completion Date

2 August 2015

Performing Laboratory

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Study Number

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SIGNATURE PAGE

DIRECT PEPTIDE REACTIVITY ASSAY (DPRA)

Initiation Date:	15 June 2015
Completion Date:	2 August 2015
Sponsor:	International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735
Sponsor's Representative:	Xiao Huang
Testing Facility:	Institute for In Vitro Sciences, Inc. 30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	Kimberly Norman August 2, 2015 Kimberly Norman, Ph.D., DABT Date
Laboratory Supervisor:	Nicole Barnes, M.S., M.B.A.

TEST ARTICLE RECEIPT

IIVS Test Article Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
15AF24	JA900-DAA	clear yellow non-viscous liquid	3 June 2015	room temperature

* - Protected from exposure to light

DIRECT PEPTIDE REACTIVITY ASSAY (DPRA)

INTRODUCTION

The Direct Peptide Reactivity Assay was used to assess the skin sensitizing potential of the test article. Synthetic peptides of cysteine and lysine were reacted with the test article for 24± 2 hours. After that incubation period the extent of peptide depletion was analyzed using High Performance Liquid Chromatography (HPLC) coupled with ultra-violet (UV) spectrometric detection.

The purpose of this study was to determine the reactivity class and the skin sensitizing potential of the test article. Percent depletion of the peptides incubated with the test article was calculated by comparing peak area of the test article incubated peptides to the mean peak area of the solvent control incubated peptides. This percent depletion was then correlated to a reactivity class and the skin sensitizing potential of the test article (negative or positive). The test article used in this assay was supplied by International Flavors & Fragrances Inc. The laboratory phase of the study was conducted from 16 June 2015 to 17 June 2015 at the Institute for In Vitro Sciences, Inc. The test article was tested in a definitive assay (in triplicate) to determine the reactivity classification and skin sensitization potential of the test article.

MATERIALS AND METHODS

Test Article Preparation

The test article was prepared at a 100mM concentration in an appropriate solvent. Calculations using the molecular weight and purity of the test article were performed to determine the appropriate amount of test article to weigh out in order to achieve approximately 3 mL of the 100 mM sample.

The test article was weighed into a prelabeled glass vial and stored at room temperature. The test article was not dissolved in the solvent until immediately before mixing with the peptides.

Test Article Solubility Test

A solubility test was performed for the test article in order to determine an appropriate solvent that completely dissolved the test article at 100 mM.

The test article was found to be soluble in acetonitrile with vortexing for approximately 1 minute.

Preparation of the Peptides

Custom synthetic peptides of lysine and cysteine (containing phenylalanine to aid in detection) were used in this assay. The purity of each peptide was 90-95%. Peptide samples were newly prepared for each sample set, and a single preparation of the peptide was used throughout the sample set. The cysteine peptide was prepared by weighing an appropriate amount of the peptide to achieve a 0.667 mM concentration in pH 7.5 phosphate buffer. The lysine peptide was prepared by weighing an appropriate amount of the peptide to achieve a 0.667 mM concentration in pH 10.2 ammonium acetate buffer. The peptide solutions were gently mixed on the shaker.

Peptide Standards

A set of serially diluted standards were prepared for each peptide. These standards were prepared by diluting the peptide solutions in dilution buffer (acetonitrile using either phosphate or ammonium acetate buffer). Six standards were prepared at concentrations of 0.534- 0.0167mM. A seventh standard was prepared containing only dilution buffer. Approximately 1 mL of each standard was pipetted into the appropriate prelabeled autosampler vials.

Controls

The positive control used in this assay was cinnamaldehyde prepared at a concentration of 100 mM. The positive control was reacted with the peptides in the same fashion as the test article. Reference controls were prepared for each solvent used in the assay. There were three sets of reference controls of acetonitrile run at different points throughout the assay. These controls consist of the solvent reacted with the peptide in the absence of test article. Co-elution controls were also prepared for the test article. The co-elution controls consisted of the test

article without the peptide. The purpose of the co-elution control was to determine if the test article elution from the HPLC column overlapped with the peptide elution.

Sample Preparation

Immediately prior to testing, the test article was diluted in the appropriate buffers to yield a 100 mM test article concentration. The test article was mixed as determined during the solubility test (vortexing/sonicating/heating). The final dosing solutions were prepared for the test article, positive control, and reference control in the prelabeled autosampler vials. Table 1 shows the make-up of the final samples for each peptide. Triplicate samples were prepared for the test article and the control. Single samples were prepared for the co-elution control.

Table 1

1:10 Molar Ratio Cysteine Peptide		**1:50 Molar Ratio Lysine Peptide**	
Amount	Solution	Amount	Solution
750 µL	Cysteine Peptide solution (or pH 7.5 phosphate buffer for Co-elution Controls)	750 µL	Lysine Peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution Controls)
200 µL	Acetonitrile	250 µL	Test Article Solution (or solvent for Reference Controls)
50 µL	Test Article Solution (or solvent for Reference Controls)		

HPLC Set-up and Operation

The separations module used in this assay was a Waters 2690/5 HPLC system. This system consists of a solvent management system for the mobile phases and a sample management system for the test article and controls. The HPLC system is coupled to a photodiode array detector set at 220 nm. The dimensions of the column used are 2.1 mm x 00 mm x 3.5 micron. The column was primed for at least two hours before the start of the assay. To prime the column, equal parts of mobile phase A (0.1% trifluoroacetic acid in HPLC grade water) and mobile phase B (0.08% trifluoroacetic acid in HPLC grade acetonitrile) were passed through the column.

Once the column was equilibrated and the samples were prepared, the autosampler vials were placed into the designated locations of the separations module carousels. The samples were incubated in the dark at 25± 2.5ºC for 24± 2 hours. After the incubation period the samples were removed from the machine and inspected for precipitate. Precipitate was not detected. The samples were returned to the machine and the testing began.

A gradient elution was used in this assay. The mobile phase changed from 10-25% acetonitrile over a 10 minute period to allow for optimal separation and gradually elute most of the sample from the column. This was followed by a rapid increase to 90% acetonitrile to remove anything remaining on the column. The column was allowed to equilibrate back to initial specs for 7 minutes between injections.

The Empower PDA software was used to convert the absorbance data from the UV detector into chromatograms of intensity versus retention time for each sample and control. At the end of the run, each chromatogram was integrated in order for the software to calculate the area under the peptide peak. Cysteine and lysine elute from the column at known times, so it was possible to determine which peaks in the chromatograms represented the peptides and use the areas under those peaks for the subsequent calculations.

Presentation of Data

The peak area calculations were obtained from the Empower PDA software and entered into the ECVAM Excel workbook. Standard curves of peak area versus concentration were prepared for each set of peptide standards. The concentrations were calculated for each sample using peak area and the equation for the appropriate standard curve. The peak area for each sample was plugged in for *y* and the equation was solved for *x* to determine the peptide concentration of the sample.

The percent depletion was calculated for each test article sample and the positive control samples as shown below.

$$\%\ Peptide\ Depletion = [1 - \frac{Test\ Article\ or\ Positive\ Control\ Peptide\ Peak\ Area}{Mean\ Peptide\ Peak\ Area\ of\ Reference\ Control\ C}] \times 100$$

Criteria for a Valid Test

In order for the assay to be considered valid, several criteria must be met. First the standard curves for each peptide must have an r^2 value greater than 0.990. The mean peptide concentration of reference control A (three replicates of acetonitrile and peptide with no test article and run with the standards) must equal 0.50± 0.05 mM.

The positive controls (100 mM cinnamaldehyde reacted with each peptide) must have a mean percent peptide depletion of 60.8-100.0% for cysteine and 40.2-69.0% for lysine among the three replicates. Also the positive control must have a standard deviation <14.9% for cysteine and <11.6% for lysine.

Reference controls B and C (run with the test article) must have a CV of peptide peak areas <15.0%. Also the mean peptide concentrations for the three replicates of each solvent used (reference control C) must be 0.50± 0.05 mM.

The standard deviations for the three replicates of the test article must meet the same criteria as the positive control for the peptide used.

Finally there must be no overlap seen between the test article peak of the co-elution control and the peptide peak. If the co-elution is seen with lysine, evaluation of the cysteine data only is acceptable. If the co-elution is seen with both peptides, the assay is considered inconclusive.

Since all of the requirements for a valid assay were achieved, the results of this assay are considered valid.

RESULTS AND DISCUSSION

The Direct Peptide Reactivity Assay

The test article was evaluated for skin sensitization potential using the Direct Peptide Reactivity Assay. The test article was reacted with synthetic peptides of cysteine and lysine and the depletion of the peptide was evaluated using HPLC with UV detection. The percent depletion of the peptides incubated with the test article was calculated by comparing peak area of the test article incubated peptides to the mean peak area of the solvent control incubated peptides. This percent depletion was then correlates to a reactivity class and the sensitizing potential of the test article (negative or positive for skin sensitization potential). The data for reactivity with each peptide is shown in Table 3 and the combined reactivity determination accounting for both peptides is shown in Table 4.

The skin sensitizing prediction models for cysteine and lysine are shown in Table 2. There are two different models which may be used; the cysteine only model may be used in the case of co-elution of the test article with the lysine peptide. This test article did not co-elute with the lysine peptide, therefore the Cysteine and Lysine model is used. The cysteine and lysine model uses the mean of cysteine and lysine percent depletions. Based on the results, the test article demonstrated minimal reactivity and predicted to be negative for skin sensitization potential.

Table 2

Mean Peptide Depletion of Cysteine and Lysine (%)		**DPRA Prediction**	**Peptide Depletion of Cysteine (%)**	
0- 6.38	Minimal Reactivity	Negative	0- 13.89	Minimal Reactivity
6.39- 22.62	Low Reactivity	Positive	13.90- 23.09	Low Reactivity
22.63- 42.47	Moderate Reactivity		23.10- 98.24	Moderate Reactivity
42.48- 100	High Reactivity		98.25- 100	High Reactivity

Table 3

Assay Date	**Peptide Tested**	**IIVS Test Article Designation**	**Sponsor Designation**	**% Peptide Depletion**	**STD**
16 June 2015	Cysteine	15AF24	JA900-DAA	9.10	1.65
	Lysine			0.34	0.58
	Cysteine	Positive Control	Cinnamaldehyde	77.48	0.94
	Lysine			64.00	1.53

Table 4

Test Article	**Average % Peptide Depletion (Cysteine and Lysine)**	**Reactivity**	**Prediction**
15AF24	4.72	Minimal	Negative

APPENDIX A

DIRECT PEPTIDE REACTIVITY ASSAY (DPRA)

1.0 PURPOSE

The purpose of this study is to evaluate the skin sensitization potential of a test article by measuring the percent depletion of a Cysteine containing peptide and a Lysine containing peptide. The reactivity of a test article with these synthetic peptides will be evaluated by combining a test article with each peptide and determining the depletion of the peptide following 24 hours of incubation at room temperature.

2.0 SPONSOR

2.1	Name:	International Flavors & Fragrances Inc.
2.2	Address:	800 Rose Lane Union Beach, NJ 07735
2.3	Representatives:	Xiao Huang

3.0 IDENTIFICATION OF TEST SUBSTANCES AND ASSAY CONTROLS

3.1	Test Article:	See Protocol Attachment 1
3.2	Assay Controls:	Positive: Cinnamaldehyde Solvent: See section 7.3

3.3 Determination of Strength, Purity, etc.

3.3.1 For GLP studies only, the Institute for In Vitro Sciences, Inc. (IIVS) will attempt to secure documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions from the Sponsor. If the Sponsor is unable to provide such information, IIVS will retain documentation supporting attempts to obtain this information with the study file and an exception will be noted in the Statement of Compliance in the Final Report.

3.3.2 IIVS will be responsible for the documentation of the analytical purity and composition of the positive and solvent controls used for the stock or working dilution of the controls. This may be accomplished by maintaining a certificate of analysis from the supplier.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Institute for In Vitro Sciences, Inc.
4.2	Address:	30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878

4.3 Study Director: Kimberly Norman, Ph.D., DABT

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 15 June 2015

5.2 Proposed Experimental Completion Date: 29 June 2015

5.3 Proposed Report Date: 24 August 2015

6.0 TEST SYSTEM

The DPRA assay utilizes an in chemico approach to measure the depletion of the Cysteine and Lysine containing peptides using high performance liquid chromatography (HPLC). The peptides are custom materials containing phenylalanine to aid in detection of either Cysteine or Lysine as the reactive center (Gerberick, et al, 2004). The concentrations of peptide within each sample or control is then measured by HPLC and UV detection at 220 nm.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of one definitive assay to determine the peptide depletion of a given test material. Three replicates of each test article will be run in the definitive trial. The percent depletion of the Cysteine and Lysine containing peptides will then be measured by HPLC after at least a 24 hr reactivity period with each test article. The percent depletion data along with the associated chromatograms will be presented.

7.1 Media and Reagents (suggested vendors in parenthesis)

7.1.1 Acetonitrile HPLC Grade (Sigma-Aldrich 439134) or equivalent (Fisher Scientific A/0626/17)

7.1.2 Lysine Peptide Ac-RFAAKAA-COOH, MW=776.2, 90-95% Purity. (JPT Peptide) (RS Synthesis)

7.1.3 Cysteine Peptide Ac-RFAACAA-COOH, MW=751.9 90-95% (JPT Peptide) (RS Synthesis)

7.1.4 Trifluoroacetic Acid 99% redistilled (Sigma-Aldrich 299537)

7.1.5 Sodium Phosphate, Monobasic Monohydrate ($NAH_2PO_4 \cdot H_2O$, CAS 7782-85-6; FW = 77.08) (Aldrich S9638)

7.1.6 Sodium Phosphate, Dibasic Heptahydrate ($NA_2HPO_4 \cdot 7\ H_2O$; FW = 268.0) (Aldrich S9390)

7.1.7 Ammonium Acetate FW = 77.08 (Sigma-Aldrich, 238074)

7.1.8 Ammonium Hydroxide 28-30% (Sigma-Aldrich, 320145)

7.1.9 Purified HPLC Grade Water (Sigma-Aldrich)

7.1.10 Cinnamaldehyde (Sigma-Aldrich, CAS 104-55-2)

7.2 Route of Administration

The test articles will initially be prepared at a 100 mM concentration. If molecular weight information is not known, then a molecular weight of 200 g/mol will be assumed. If the purity information is not known, then 100% purity is assumed to prepare the test materials. The test articles will be diluted in glass vials in the appropriate solvent prior to HPLC testing.

The stability of the test article(s) under the storage conditions at the testing facility and under the actual experimental conditions will not be determined by the Institute for In Vitro Sciences, Inc. (IIVS).

7.3 Controls

Positive control: Cinnamaldehyde will be diluted in acetonitrile at 100 mM.

Solvent Control: The following solvent choices can be used for diluting the test articles:

7.3.1 Acetonitrile
7.3.2 Water
7.3.3 1:1 Acetonitrile:Water
7.3.4 Isopropanol
7.3.5 1:1 Acetonitrile:Acetone
7.3.6 1:10 DMSO:Acetonitrile (if this mixture does not work a 1:1 DMSO:Acetonitrile solution mix can be attempted)

The appropriate solvent control must be run with each run sequence.

7.4 Solubility Compatibility Testing

Prior to testing in the assay, the solubility of the test article at a concentration of 100 mM in acetonitrile or appropriate solvent must be determined. When possible this solubility determination should be performed prior to the start of the assay. **Acetonitrile is the solvent of choice for the test article dilution.**

For some test articles, an intermediate solvent may be used to produce a solution of the test article. In this case, the test article may be dissolved in HPLC grade deionized water, a 1:1 mixture of Water:Acetonitrile, Isopropanol, Acetone, a 1:1

mixture of Acetone:Acetonitrile, or a 1:10 mixture of Dimethylsulfoxide (DMSO):Acetonitrile.

Note: The column used in this assay will not work properly with test materials that are not soluble. Any test material that does not go into solution or a homogenous suspension should not be run through the HPLC machine. Heating, sonication, and/or filtration should be attempted for test materials that are not soluble in efforts to remove particulates. Applying suspensions, slurries, particles, semi-viscous liquids, etc., may result in clogging of the HPLC machine and irreparable damage to the column. If this occurs the machine will be marked OUT OF SERVICE. However, if it is decided that the test article is not suitable for the assay system the Sponsor will be contacted.

7.5 Preparation and Delivery of Test Article

Each test chemical will be pre-weighed into a sterile glass vial. Test chemicals will be dissolved into approximately 3.0 mL of the appropriate solvent as determined during the solubility testing to prepare a 100 mM solution. The amount of the test chemical to be weighed out is determined based on molecular weight (MW) and purity. If no purity information is available, assume 100% purity. The calculation below can be used to determine the amount of test article to be weighed and the amount of solvent needed to obtain a 100 mM solution.

$$3mL \times \frac{1\,L}{1000mL} \times \frac{100\,mmoles}{L} \times MW\left(\frac{mg}{mmole}\right) \times \frac{100}{\%\,Purity} = \text{Target Weight (mg)}$$

The molecular weight, purity, and actual weight of the test article will be recorded in the study workbook.

Make sure the glass vials containing the test articles or associated controls are capped and stored under the appropriate conditions until testing is to be performed. Storage conditions will be supplied by the supplier/sponsor. If no conditions are provided the test article will be stored in the laminar flow hood in the capped vial at room temperature until the samples are placed in the HPLC machine. **Do not add the solvent to the test article until the assay is ready to begin.**

Once the assay is all set up and ready to begin then the ~3mL (or appropriate) amount of solvent can be added to make the 100 mM solutions to be incubated with each peptide. Once the 100 mM solutions are mixed with the peptides the 24 ± 2 hour incubation begins.

7.6 Preparation and Delivery of Control Articles

Positive Control: 100 mM sample of the positive control Cinnamaldehyde will be

prepared as described for the test articles in section 7.5.

Reference Controls: The reference controls is a peptide solution where the test article is replaced by the solvent used to dissolve the test article. Therefore, reference controls are prepared as described in Table 1.

Co-Elution Controls: Co-elution controls are also prepared as described in Table 1. The appropriate buffer solution is used in place of the peptide solution.

DO NOT ADD BUFFERS TO PEPTIDES UNTIL READY TO BEGIN THE ASSAY

Cysteine Peptide 0.667 mM (0.501 mg/mL): Weigh an appropriate amount of Cysteine containing peptide into an appropriate container. The assay requires ~800 µL per test sample, therefore, be sure to prepare enough solution to be used for all samples being tested. All samples should be prepared with the same batch of peptide. For example, to prepare 25 mL of solution, weigh 12.5 mg of cysteine containing peptide.

Lysine Peptide 0.667 mM (0.518 mg/mL): Weigh an appropriate amount of Lysine containing peptide into an appropriate container. The assay requires ~800 µL per test sample, therefore, be sure to prepare enough solution to be used for all samples being tested. All samples should be prepared with the same batch of peptide. For example, to prepare 25 mL of solution, weigh 13.0 mg (12.95 mg) of Lysine containing peptide.

7.7 Preparation of Solutions

Label three autosampler vials for each test article and control with the appropriate number designation based on where they will be located in the HPLC carousel that corresponds to the test article(s). When ready to perform the assay, dissolve the appropriate test article or control by adding the appropriate amount of solvent. The resulting solution should yield a 100 mM concentration (see calculation in section 7.5). Mix the solution as determined during the solubility testing (i.e. vortexing and/or sonicating if necessary) and verify that the test article is in solution form.

7.7.1 Preparation of Peptide Solutions

Cysteine Peptide: Based on the calculation of the amount of peptide needed (section 7.6), once the actual samples are weighed; the appropriate amount of **pH 7.5 phosphate buffer** solution can be added to the cysteine containing peptide. The following calculation can be used to determine the correct amount of buffer needed based on the actual weight of the peptide:

$$\text{mL pH 7.5 Buffer} = \frac{\text{mg Peptide}}{0.501\ \text{mg/mL}}$$

Lysine Peptide: Based on the calculation of the amount of peptide needed

(section 7.6), once the actual samples are weighed the appropriate amount of **pH 10.2 Ammonium Acetate buffer** solution can be added to the Lysine containing peptide. The following calculation can be used to determine the correct amount of buffer needed based on the actual weight of the peptide.

$$\text{mL pH 10.2 Buffer} = \frac{\text{mg Peptide}}{0.518\ \text{mg/mL}}$$

7.7.2 Preparation of Standard

The standards are prepared in a solution of 20% Acetonitrile:Buffer. The Peptide stocks will be serially diluted from a concentration of 0.534 mM to 0.017 mM. The standards will be diluted in dilution buffer specific for each peptide. The dilution buffer can be prepared as listed below:

Dilution Buffer: 8 mL of Buffer (pH 7.5 phosphate buffer for Cysteine, or pH 10.2 ammonium acetate buffer for Lysine) is mixed with 2 mL of acetonitrile. This ratio can be adjusted based on the amount of buffer needed but should remain as 20% acetonitrile:buffer.

The preparation of the serial dilution will begin by mixing 1600 µL of the previously prepared peptide stock at a concentration of 0.667 mM with 400 µL of acetonitrile to achieve a concentration of 0.534 mM (Dose ID 1 in Figure 1). The remainder of the doses will be serially diluted as shown in Figure 1. The last vial (Dose ID 7 in Figure 1) will only contain the dilution buffer. Once the standard has been serially diluted, approximately 1 mL of each standard concentration will be added to an autosampler vial for testing.

Figure 1: Serial Dilution of Standard

Dose ID			TA				Solvent	Final Concentration	
1	1600	µL	**Peptide Stock** (0.667mM)	+	400	µL	Acetonitrile	0.534	mM
2	1.0	mL	1	+	1.0	mL	Dilution Buffer	0.267	mM
3	1.0	mL	2	+	1.0	mL	Dilution Buffer	0.133	mM
4	1.0	mL	3	+	1.0	mL	Dilution Buffer	0.067	mM
5	1.0	mL	4	+	1.0	mL	Dilution Buffer	0.033	mM
6	1.0	mL	5	+	1.0	mL	Dilution Buffer	0.017	mM
7	NA		NA	+	NA		Dilution Buffer	0.000	mM

7.8 Definitive Assay

Samples will be prepared in triplicate for the definitive assays. If only one HPLC machine is available and more than six test articles are to be run then the Cysteine and Lysine assays will be run on separate days. The following steps should be performed to set up the samples for testing.

1. Assemble all prepared reagents, solvents, and solutions:
   a. Peptide Stock Solutions
   b. Buffers
   c. Acetonitrile
   d. Test Articles
   e. Appropriate solvents and Reference Controls

2. Using 1-2 mL autosampler vials, prepare the final sample solutions by adding the appropriate peptide solution (see the Table 1).

3. Cap the vials and place them in the HPLC autosampler at room temperature for approximately 24 hours. HPLC analysis of the batch of samples should start 24 ± 2 hours after the test chemical was added to the peptide solution.

Table 1: Preparation of Final Dosing Solutions

1:10 Ratio Cysteine Peptide		1:50 Ratio Lysine Peptide	
Amount	Solution	Amount	Solution
750 µL	Cysteine Peptide solution (or pH 7.5 phosphate buffer for Co-elution Controls)	750 µL	Lysine Peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution Controls)
200 µL	Acetonitrile	250 µL	Test Article Solution (or solvent for Reference Controls)
50 µL	Test Article Solution (or solvent for Reference Controls)		

7.9 HPLC Operation and Analysis

HPLC analysis is performed using a flow of 0.35 mL/min and a linear gradient from 10-25% Acetonitrile over 10 minutes followed by a rapid increase to 90% acetonitrile to remove any other materials. The HPLC system should be programmed to inject an equal volume of each test article, standard and control. The injection volume should be 6 µL, however, if peaks are determined to be too broad or too narrow any volume between 3-10 µL may be used (injection volume depends on the HPLC system being used). The mobile phases are listed under Table 2. The HPLC system should be programmed as outlined in Table 3.

Table 2: Preparation of Mobile Phases

Reagent	Preparation
Mobile Phase A	Add 1.0 mL of Trifluoroacetic acid to 1 L of HPLC grade water.
Mobile Phase B	Add 850 µL of Trifluoroacetic

	acid to 1 L of HPLC grade acetonitrile.

Table 3: HPLC Conditions

Column Temperature	**30±3°C**			
Sample Temperature	**25°C (room temperature)**			
Detector	**220 nm**			
Injection Volume	**6 µL*** (set the autosampler needle path to avoid drawing from the bottom of the autosampler vial)			
Run Time	**20 minutes**			
Flow Conditions	**Time**	**Flow**	**%A**	**% B**
	0 min	0.35 mL/min	90	10
	10 min	0.35 mL/min	75	25
	11 min	0.35 mL/min	10	90
	10 min	0.35 mL/min	10	90
	13.5 min	0.35 mL/min	90	10
	20 min	End run	NA	NA

* - This may be adjusted based on the sensitivity of the HPLC being used. If peaks are too broad then this can be updated.

7.8.1 HPLC Analysis Sequence

Prepare two separate analysis sequences. The first sequence should consist of the calibration standards, Reference Controls, and Co-elution controls. The first sequence can be run as soon as all standard and control samples have been prepared for analysis or any time prior to the initial injection of the test articles. An example run has been included in Table 4.

Table 4: Run Sequence 1

Sample	Notes
STD 1	Verify linearity of response Verify precision and accuracy of pipetting Mean peptide concentration of Reference Controls A = 0.50 +/- 0.05 mM
STD 2	
STD 3	
STD 4	
STD 5	
STD 6	
STD 7 (dilution buffer)	

Reference Control A, rep 1	
Reference Control A, rep 2	
Reference Control A, rep 3	
Co-elution Control 1	Verify co-elution of test chemicals with peptide
Co-elution Control 2	
Co-elution Control 3	
Reference Control B, rep 1	Verify stability of Reference Controls over analysis time
Reference Control B, rep 2	
Reference Control B, rep 3	

The second analysis sequence should consist of the assay controls, solvent controls, test materials and co-elution controls (if needed).

7.8.2 Co-Elution Controls

In some cases a test material will co-elute with the peptides which interferes with the integration of the peptide peaks. Co-elution controls are run in efforts to help distinguish the peaks and calculate the percent depletion.

7.9 Data Analysis and Calculations

The concentration of peptide is determined in each sample from absorbance at 220 nm measuring the peak area of either the Cysteine or Lysine containing peptide peaks and calculating the concentration of peptide using the linear calibration curves derived from the standards. The peak area data and chromatograms will be done using the Empower™ Software. The calculation of the standard curve and percent depletion will be done using Excel software.

The percent depletion of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area and dividing that by mean peak area of the reference controls (or solvent controls).

1. Integrate the appropriate peaks in the Empower™ Software. Once the peaks are integrated, the Empower™ Software will generate the peak area for standards, samples and controls. The peak area of each integrated peak must be reported. The peaks should be consistently integrated "valley to valley". There may be some instances when this is not practical, but should be appropriate for most chromatograms.

2. Once the peaks are integrated then the peak data for the standards, test articles, and controls will be printed.

3. The peak area's will then be entered in Excel, using the ECVAM excel template (filename boilerplate_DPRA_results.xls). The peak areas for the standards will be entered and Excel will generate a linear calibration curve based on the concentration of standards and the peak area. Acceptable calibration curves will have an $r^2 > 0.990$. Calculate the mean peptide

concentration in Reference Controls A., SD and CV. The mean should be 0.50 +/- 0.05 mM.

4. Once the peak areas for the reference controls are entered the mean peptide peak area at 220 nm for the nine Reference Controls in acetonitrile, SD and CV will be calculated. The CV must be <15.0%.

5. Calculate the mean peptide peak area at 220 nm for the three Reference Controls for any additional solvents other than acetonitrile.

6. Calculate the mean peptide concentration (mM) for the three additional Reference Controls for each additional solvent used, SD and CV. The mean should be 0.50 +/- 0.05 mM.

7. UV absorbance is a general detection method and interfering peaks may occur. If there is uncertainty regarding the identity of the peak, verify the UV absorbance spectrum and retention time are consistent with the appropriate reference controls (based on the specific solvent used).

8. Some test chemicals will co-elute with the Cysteine or Lysine peptides. In order to detect possible co-elution of the test chemicals with a peptide, the test articles included in the run will be injected alone ("Co-elution Controls") during the run sequence. The chromatograms obtained from either additional testing method will then be compared to the three replicates of the appropriate reference control. If the test article co-elution control absorbs at 220 nm and has a similar retention time as a peptide (overlap of "valley to valley" integration periods), then co-elution of the test chemical with that peptide should be reported. In order to assure that baseline noise is not being called interference, the "interfering" chemical peaks should have a peak area that is >10% of the mean peptide peak area in the appropriate reference control.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if all of the following acceptance criteria are met:

8.0.1 System Suitability

The calibration linearity must have an $r^2 > 0.990$ and the mean peptide concentration of the Reference Control A must equal 0.50 +/- 0.05 mM.

8.0.2 Positive Control

The positive control range is still under development at IIVS. Until enough data has been collected the ECVAM validation ranges will be used to determine the acceptable range of the positive control. The mean percent peptide depletion value of the three replicates for Cinnamaldehyde will then be based on the ranges in the following table.

Table 5: Positive Control Acceptance Range

	% Cysteine Depletion		% Percent Lysine Depletion	
	Lower Bound	*Upper Bound*	*Lower Bound*	*Upper Bound*
Cinnamaldehyde (Positive Control)	60.8	100.0	40.2	69.4

The positive control must also meet the flowing standard deviation criteria in Table 6:

Table 6: Standard Deviation of Replicates

Peptide	Standard Deviation of Percent Depletion
Cysteine Depletion	must be < 14.9%
Lysine Depletion	must be < 11.6%

8.0.3 Reference and Solvent Control Stability

The CV of the peptide peak areas for the nine total reference controls B and C in acetonitrile must be < 15.0%. For each solvent used, the mean of the peptide concentration of the three appropriate reference controls must be 0.50 +/- 0.05 mM.

8.0.4 Test Article Acceptance Criteria

The standard deviation for each test article must meet the same criteria as established for the positive control in section 8.0.2.

9.0 EVALUATION OF TEST RESULTS

9.0.1 System Suitability (Assay Standards)

The use of the assay standards is to determine the functionality of the system on a given test day for each peptide. The assay standards should present a linear calibration curve with an r^2 value > 0.990 using the peak area values at each concentration of the standards.

Figure 2: 1:10 Cysteine and 1:50 Lysine Prediction Model

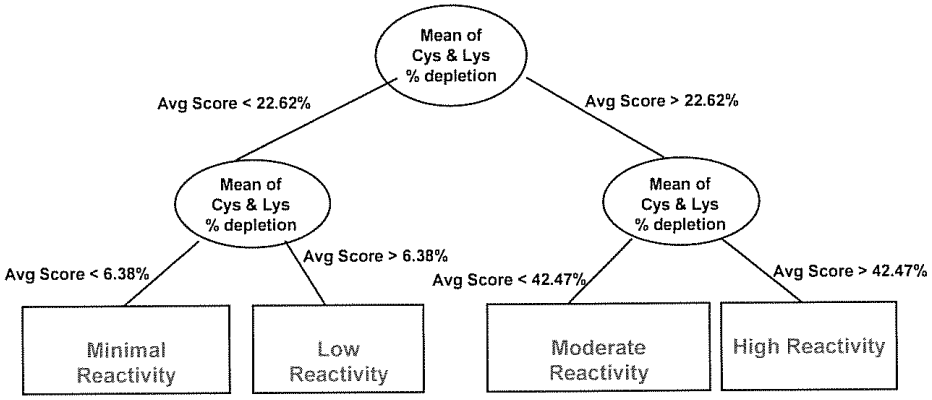
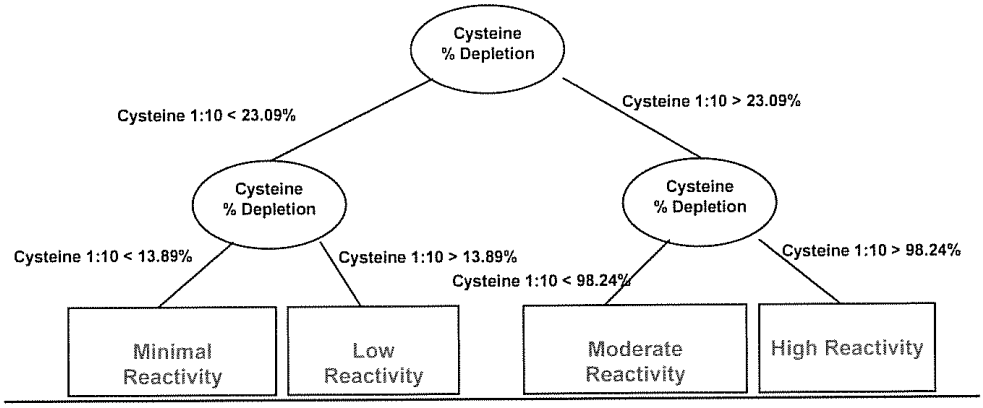


Figure 3: 1:10 Cysteine Only Prediction Model



10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A copy of the protocol used for the study, any amendments and any significant deviation(s) from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 PROTOCOL AMENDMENTS

When it becomes necessary to change the approved protocol for a specific study, the change and the reason for it should be put in writing and signed by the Study Director as soon as practical. When the change may impact the study design and/or execution, verbal agreement to make this change should be made between the Study Director and Sponsor. This document is then provided to the Sponsor and is attached to the protocol as an amendment.

13.0 REFERENCES

Gerberick, G.F., et al. (2004) Development of a peptide reactivity assay for screening contact allergens. Toxicological Sciences. 81: p. 332-343.

14.0 APPROVAL

See sample submission form dated 28 May 2015
SPONSOR REPRESENTATIVE DATE

Xiao Huang
(Print or Type Name)

Kimberly Norman June 15, 2015
IIVS STUDY DIRECTOR DATE

PROTOCOL ATTACHMENT 1

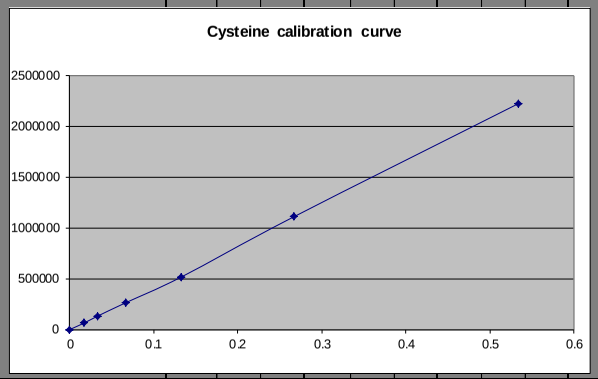
IIVS Test Article Designation	Sponsor Designation	Molecular Weight (average)
15AF24	JA900-DAA	1082.0 g/mol

REGULATORY REQUIREMENTS:

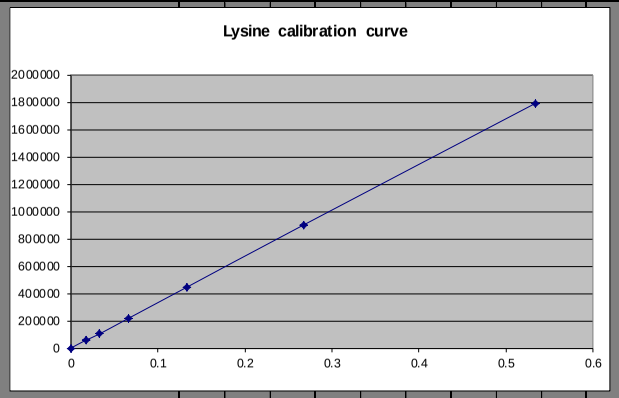
Will this study be conducted according to **GLPs**? [ ] **YES** or [X] **NO**
If **YES**, please indicate which agency(ies) guidelines are to be followed:
[ ] FDA; [ ] EPA TSCA; [ ] EPA FIFRA; [ ] OECD; [ ] Other _____

APPENDIX B

Vehicle	Code	Conc/ replicate	Cysteine													Reactivity Class (CYS only)	Criterion	Criterion met?
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Co-elution			
	STANDARD																	
		STD1	2227200	0.534													Cysteine: r2 > 0,99	Cys R2 0.999758651 **YES**
		STD2	1112840	0.267														Cys Intercept -11267.67122
		STD3	518795	0.133														Cys Slope 4185619.776
		STD4	264593	0.067														
		STD5	131732	0.033														
		STD6	63797	0.017														
		Dil Buff	0	0														
	REF CTRL A																	
		r1	2037889	0.489570668						**0.49**	**0.00**	**0.01**					Range Mean Conc Cys [0.45 to 0.55]	**YES**
		r2	2059840	0.494815053														
		r3	2056643	0.494051247														
	REF CTRL B																	
		r1	1878773	0.451555749														
		r2	1885243	0.453101517													Peak Area CV ctrl B < 15% - Cys	**YES**
		r3	1873980	0.450410637			**1871590**	**14026**	**0.0075**	**0.450**	**0.003**	**0.01**						
		r4	1847631	0.444115512														
		r5	1881058	0.452101665														
		r6	1862854	0.447752489														
Acetonitrile	**REF CTRL C**																Peak Area CV ctrl C < 15% - Cys	**YES**
		r1	1862768	0.447731942														
		r2	1882715	0.452497545			**1878474**	**14074**	**0.0075**	**0.45**	**0.0034**	**0.0074**					Range Mean Conc Cys [0.45 to 0.55]	**YES**
		r3	1889940	0.454223693														
Acetonitrile	**POSITIVE CONTROL**																	
	Cinnamic Aldehyde	r1	443295	0.108601043	76.40132782	76.40132782											60,8 < Mean % Depl Cys < 100	**YES**
		r2	412503	0.101244426	78.04053041	78.04053041	**422982**	**17594**	**0.0416**	**0.1037**	**0.0042**	**0.0405**	**77.483**	**0.9366**	**0.0121**			
		r3	413149	0.101398764	78.0061408	78.0061408											SD % Depl Cys < 14,9	**YES**
Acetonitrile	**15AF24**																	
	15AF24	r1	1732303	0.416562126	7.78138571	7.78138571											SD % Depl Cys < 14,9	**YES**
		r2	1717465	0.413017131	8.571282049	8.571282049	**1707560**	**30910**	**0.0181**	**0.4107**	**0.0074**	**0.018**	**9.0986**	**1.6455**	**0.1809**	N	MINIMAL	
		r3	1672911	0.40237259	10.94310046	10.94310046												



Vehicle	Code	Conc/ replicate	Lysine														Mean Depletion	Reactivity Class	Criterion	Criterion met?
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area			Peptide Conc			Peptide Depl.			Co-elution				
							mean	SD	CV	mean	SD	CV	mean	SD	CV			(CYS + LYS)		
	STANDARD																			
		STD1	1792456	0.534															Lysine: r2 > 0,99	Lys R2 0.999977007 **YES**
		STD2	900130	0.267																Lys Intercept -2288.684013
		STD3	445044	0.133																Lys Slope 3363925.752
		STD4	219705	0.0667																
		STD5	104066	0.033																
		STD6	57055	0.017																
		Dil Buff	0	0																
	REF CTRL A																			
		r1	1615610	0.480955527						0.4807	0.0044	0.0092								
		r2	1599571	0.476187586																
		r3	1629280	0.485019232															Range Mean Conc Lys [0.45 to 0.55]	**YES**
	REF CTRL B																			
		r1	1646811	0.490230702																
		r2	1631443	0.48566223																
		r3	1653397	0.492188534			**2E+06**	**77840**	**0**	**0.5103**	**0.0231**	**0.0453**								
		r4	1780232	0.529892993																
		r5	1777617	0.529115627																
		r6	1796823	0.534825028															Peak Area CV ctrl B < 15% - Lys	**YES**
Acetonitrile	REF CTRL C																			
		r1	1676383	0.499021622															Peak Area CV ctrl C < 15% - Lys	**YES**
		r2	1624572	0.483619676			**2E+06**	**71477**	**0.0423**	**0.5028**	**0.0212**	**0.0423**								
		r3	1765862	0.525621198															Range Mean Conc Lys [0.45 to 0.55]	**YES**
Acetonitrile	POSITIVE CONTROL																			
	Cinnamic Aldehyde	r1	608097	0.181450403	63.99532488	63.99532488														
		r2	582208	0.173754336	65.52818071	65.52818071	**608039**	**25802**	**0.0424**	**0.1814**	**0.0077**	**0.0423**	**63.999**	**1.5277**	**0.0239**				40,2< Mean % Depl Lys < 69,4	**YES**
		r3	633811	0.189094448	62.47283058	62.47283058														
																			SD % Depl Lys < 11,6	**YES**
Acetonitrile	15AF24																			
	15AF24	r1	1671962	0.497707383	1.00518728	1.00518728														
		r2	1709658	0.508913338	-1.226746496	0	**2E+06**	**31828**	**0.0187**	**0.5077**	**0.0095**	**0.0186**	**0.3351**	**0.5803**	**1.7321**	N	5	MINIMAL		
		r3	1735232	0.516515765	-2.740951568	0													SD % Depl Lys < 11,6	**YES**



APPENDIX C

IFF

INTERNATIONAL FLAVORS & FRAGRANCES (IFF R&D) 1515 HIGHWAY 36, UNION BEACH, NJ 07735 (732) 264-4500

CREATORS AND MANUFACTURERS OF FLAVORS, FRAGRANCES AND AROMA CHEMICALS CABLE: INTERIFF NEW YORK

Certificate of Analysis

JA900-DAA; Jeffamine ED900 diacrylamide; lot RDLV28986.

JA900-DAA, lot RDLV28986, is 51.4% pure, has M_n = 1082 and meets all analytical standards set by International Flavors & Fragrances, Inc. The expiration date is 3/2017.

This material should be stored ambient, protected from light.

Lisa Veliath, PhD
Research Investigator
International Flavors & Fragrances
1515 Highway 36
Union Beach, NJ 07735
732-335-2871

March 20, 2015